Reference: *Biol. Bull.*, 144: 180–191. (February, 1973)

DEMONSTRATION OF AN ACTION OF ACETYLCHOLINE ON THE CENTRAL NERVOUS SYSTEM OF A CRAB

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A question which has remained unsettled for some time concerns the function of the acetylcholine (ACh) found in crustacean nervous systems. The most likely role for this compound is that of a synaptic transmitter agent in the central nervous system but of those criteria listed by Paton (1958) for evaluation of suspected transmitter agents, one has resisted experimental verification: administration of ACh to the central nervous system has not consistently produced effects which mimic the action of a natural transmitter. Although Bonnet (1938) reported the demonstration of such an effect of ACh on the crayfish, neither an extensive study by Prosser (1940) nor more recent attempts (Welsh, 1961) confirmed the earlier study.

The purpose of this report is to show that small doses of ACh, when added to a crab's central nervous system, do indeed have definite effects and moreover these effects are influenced by eserine and atropine. The present results support the contention that ACh is a synaptic transmitter agent in the central nervous system of crabs.

Materials and Methods

The crab *Carcinus maenas* was used for these experiments. The physiological saline solution (Fatt and Katz, 1953) contained (mM) NaCl (513), KCl (13), $CaCl_2$ (12), and $MgCl_2$ (24). The saline was buffered at pH 7.4 by 5 mM Tris-Maleate. The drugs (acetylcholine chloride, atropine sulfate, and eserine salicylate) were obtained from Sigma Chemical Company. Stock solutions were made up in distilled water and stored in the refrigerator until use. These stocks were made up frequently. All experiments were done at room temperature, 22–25° C.

Two types of experiments were performed. The first type involved perfusion of the thoracic ganglion through the sternal artery and recording the mechanical activity of the legs in response to ACh. The administration of drugs to the central nervous system *via* the arterial system was necessary for the success of the experiments. Although crustaceans have open circulatory systems, there is a well-defined vascular system in the thoracic and supra-esophageal ganglia (Sandeman, 1967). The second type involved adding the drug directly to the ganglion by electroosmotic injection from a glass micropipette and recording the induced electrical activity in the neuropile by means of a second, adjacent, micropipette.

For the experiments which required the recording of mechanical activity the crab was tied, with rubber bands, to a small Plexiglas platform. The crab's legs hung over the edge of the platform and thus could move freely. The crab was held

in air over a large dish which was used to catch the perfusate dripping out of the animal. The carapace over the heart was removed and, with the aid of a stereo microscope, the dorsum of the heart was removed and the blood rinsed out with saline. A small polyethylene cannula was then inserted into the orifice of the sternal artery in the ventral part of the heart; the artery was tied to the cannula with silk suture, and a slow perfusion of saline from a Mariotte flask was started. ACh was injected in aliquots of one ml while eserine and atropine were perfused through continuously from separate flasks. In this preparation, mechanical activity of the legs was monitored on a Grass Model 7 Polygraph. This was achieved by attaching one or more legs with thread at convenient places, to a Grass FT.03 strain gauge.

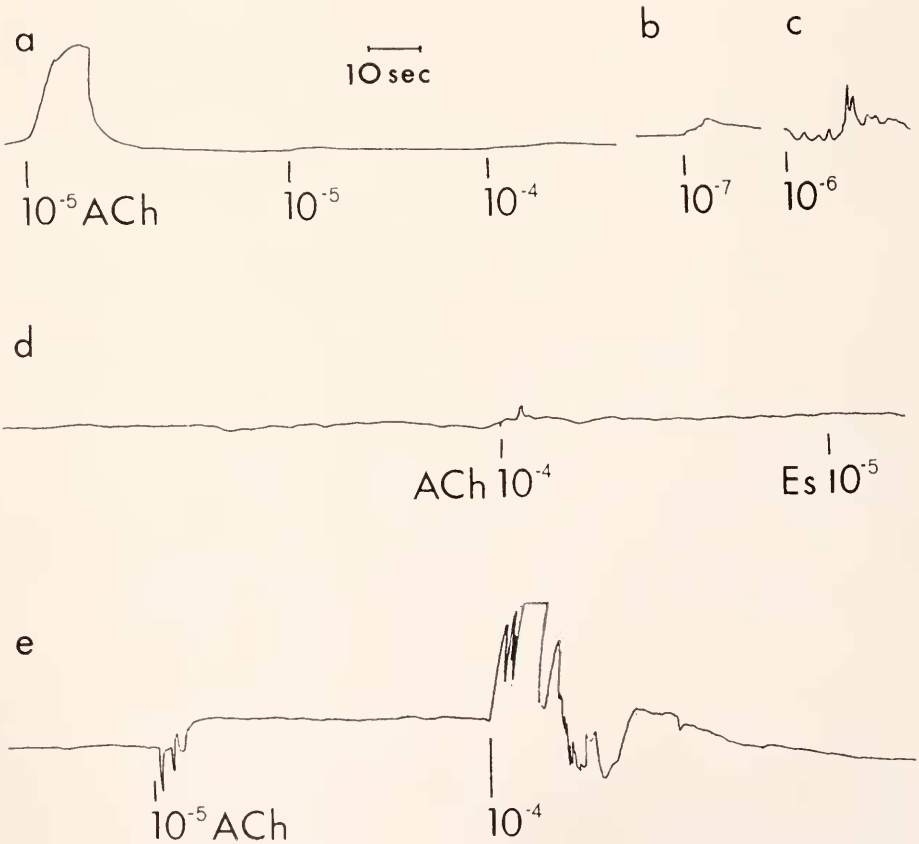

FIGURE 1. The effect of acetylcholine and eserine on leg movements of *Carcinus*. In (A) the injection of one ml of ACh-saline (10^{-5} g/ml) into the perfusion line elicited a transient leg movement. Subsequent application of the drug at the same and at a greater concentration showed no notable effect. In (B) and (C) the responses of two different crabs to lower concentrations of acetylcholine are shown. In (D) the response of a fourth crab to an injection of acetylcholine (10^{-4} g/ml) is displayed and in (E) the responses of the same crab at a lower and at the same concentration as before but both in the presence of eserine (10^{-5} g/ml).

For the second type of experiment, the crab's legs were removed at the point of autotomy, and the crab was tied to the platform ventral side up. The tail, ventral exoskeleton, and underlying musculature were then removed in conjunction with copious rinsing in seawater. The thoracic ganglion thus exposed was kept moist by topical application of the saline solution. Micropipettes with a tip diameter of less than 0.5 microns were drawn from Pyrex tubing using the Industrial Sciences puller. One micropipette, used to inject ACh, contained only ACh (1–10 mM) in distilled water. The second pipette, used to record electrical changes in the neuropile, was filled with 3M KCl and had a resistance of 5–15 megohms. A Ag : AgCl electrode in the fluid next to the ganglion completed the circuits. The mode of recording was single-ended, and the amplifier used was a Bioelectric Instruments NF1. The neuropile potentials were observed on one channel of a Tektronix 561 oscilloscope and were photographed with a Grass Kymograph camera on a "slave" oscilloscope. The pipette used to deliver the ACh was connected to a Tektronix 161 pulse generator through an operational amplifier circuit designed to insure constant currents, despite large changes which might be encountered in the resistance of the micropipettes. A description of this current-clamp device is being prepared for publication (G. Katz, in preparation). The second channel of the oscilloscope was used to monitor the current pulse during the electroosmotic injection. The method of electroosmotic injection (Krnjevic and Whittaker, 1965; Chiarandini, Reuben, Brandt and Grundfest, 1970) is attractive because of its ease of use and simplicity; for quantitative studies however, iontophoretic application would be preferable.

The pipettes were held by the same micromanipulator, a Narishige MD-2, which allowed independent positioning of the tips to within 0.2 mm. The spacing was within 0.2 mm at the start but after insertion it was probable that the spacing changed. By selective probing of the ventral surface of the ganglion, areas in the neuropile were found which responded to ACh with electrogenesis. Many insertions showed no response. No intracellular recordings were made and no responses were recorded from points near the surface. No systematic study was made of the frequency or location of responsive sites within the neuropile because the intent was qualitative; that is, to determine whether or not direct injection of ACh would produce an effect.

Results

The effect of acetylcholine

Figure 1 depicts the result obtained upon addition of one ml of saline containing ACh to the perfusion line. Three different preparations with different response sensitivities are shown in Figure 1A, B, and C. I found that the sensitivity of the crab to ACh diminished with time; if the application of ACh was made within a few minutes after cannulation, concentrations as low as 10^{-8} g/ml injected into the perfusion line could cause leg movement. After an hour of perfusion and with no other treatments, 10^{-6} g/ml or more were required. In no instance was a concentration greater than 10^{-4} g/ml necessary.

I also found that repeated application of ACh resulted in diminished sensitivity. An example of this is shown in Figure 1A where a second application of 10^{-5} g/ml

was ineffective and even 10^{-4} g/ml showed little effect. This decrease in sensitivity was also observed with the electroosmotic injection method and it will be discussed later.

Finally, as noted by many previous investigators, topical application of ACh in concentrations as high as 10^{-3} g/ml was ineffective. This method of application involved dripping the solution directly on the ganglion.

The effect of eserine and atropine

Eserine by itself caused leg movements of low magnitude but it was easily shown that the effect of ACh were enhanced during eserine treatment. Figure 1D and E shows this potentiation. While there was relatively little control response to 10^{-4} g/ml ACh, upon the addition of eserine, even a ten-fold reduction in dosage elicited an easily-recorded response and a repeat of the control dose caused vigorous and prolonged leg movements. Discontinuation of perfusion with eserine resulted in a loss of this heightened responsiveness to ACh. In connection with these observations, spontaneous increases in sensitivity to ACh were never observed. Instead, the common observation was, as noted above, a decrease in sensitivity. Thus, the result to be emphasized is the increase in sensitivity during eserine treatment rather than the decrease in sensitivity after eserine perfusion was discontinued.

Eserine also caused an increased responsiveness to mechanical stimulation. Normally there were no spontaneous movements; the crab, after several minutes perfusion, remained limp and unresponsive to moderate tapping on the rostrum. In contrast, the eserine-treated animal responded to tapping on its rostrum with extensive leg movements. Figure 2A depicts this effect.

Atropine reduced the response to ACh. Figure 2B and C displays an example of this effect. After the addition of atropine, the response to ACh was recorded and then, after extensive perfusion with normal saline, another response was recorded and was found to be considerably greater. The effect of atropine was difficult to reverse and in this case, 45 minutes perfusion was necessary. Diminished sensitivity to repeated applications of ACh made it difficult to establish a control response before the addition of this drug, which also attenuated the response. For this reason, in this instance, I chose not to test the animal before the addition of atropine in order to work with as fresh a preparation as possible. Since I have never observed a spontaneous increase in sensitivity to ACh, it seems reasonable to ascribe the larger response in Figure 2C to the absence of atropine.

Electroosmotic injection of acetylcholine

Since the sternal artery passes through the thoracic ganglion and branches to form an artery for each leg, it is conceivable that the effect of ACh obtained by perfusion is initiated peripherally and not in the central nervous system. A direct test of this hypothesis is obviously desirable. The change in electrical activity following electroosmotic injection of ACh into the neuropile is shown in Figures 3 and 4.

In Figure 3, records taken from four different insertions (two animals) are shown. The voltage record (top trace) before the pulse is stable but upon initiation of electroosmotic flow there is considerable electrical activity. The interpretation

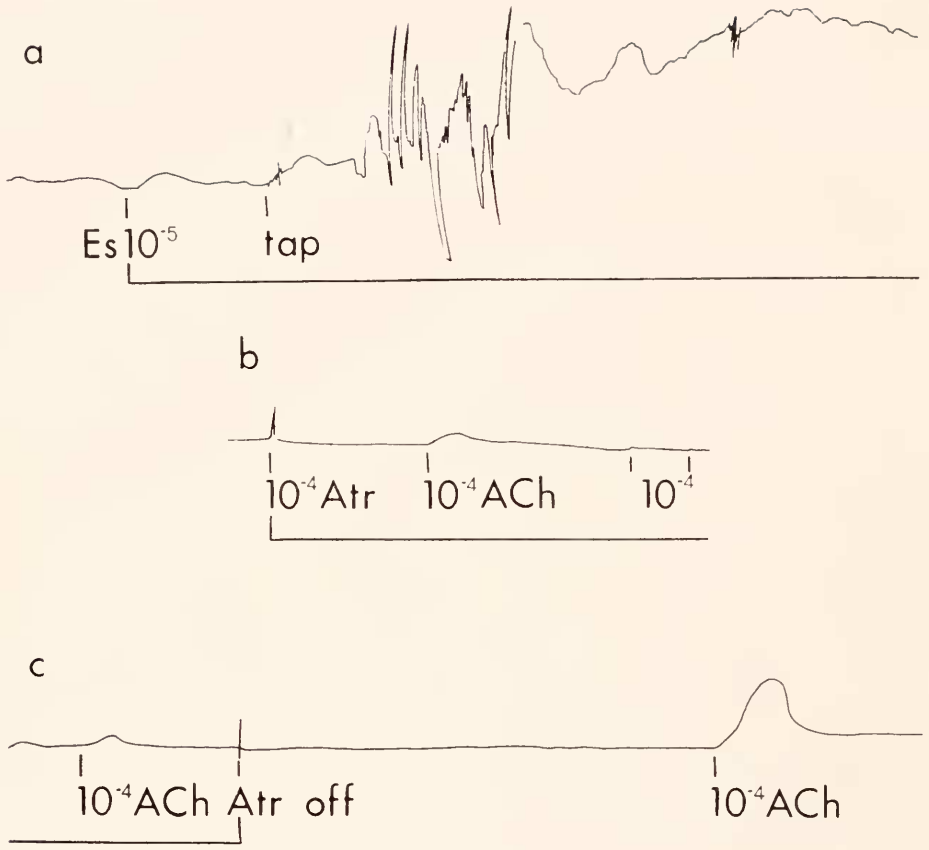

FIGURE 2. The influence of eserine on mechanical stimulation and of atropine on the acetylcholine effect. In (A) the leg movements in response to a sharp rap on the rostral carapace after the perfusion of eserine. Normally the crabs are flaccid and do not respond to such stimulation. In (B) atropine (10^{-4} g/ml) was added before the acetylcholine and a small response was noted. In (C) the response was depressed until after removal of the atropine by extensive perfusion.

of these potential changes is that they represent current flow through the resistance formed by the extracellular fluid lying between different neuropile elements; it is probable that both synaptic and axonal responses contribute, the latter in response to the former. It is unlikely that the current flow from the delivery pipette itself caused the observed effects because the majority of attempts resulted in no electrical response at all. Figure 3 also shows that the electrical activity subsided toward the end of the pulse even though the current pulse remained steady. This can probably be attributed to desensitization of the postsynaptic element. Another fact which suggests that the primary effect is on synaptic rather than electrically excitable membrane is that the effect of ACh was graded; the size of the response grew with increase in electroosmotic current.

Repeated application of ACh resulted in diminished responses. Figure 4A shows again that the acitivity subsided with time during one pulse. Figure 4B, C, and D all show that repetition of the pulse at short intervals could attenuate or even abolish the response. The repeated pulses are superimposed. If the interval between pulses was on the order of a few seconds, this diminished response was not observed; this can be another manifestation of desensitization. The presence of graded activity and of desensitization argue further against the possibility that the current flow itself could cause these neuropile potentials, since synaptic membrane is electrically inexcitable (Grundfest, 1957).

I would like to emphasize that the important point of this section is that the effect of ACh can be localized to the thoracic ganglion. Even though the possibility of an effect of ACh on peripheral elements of the nervous system was not

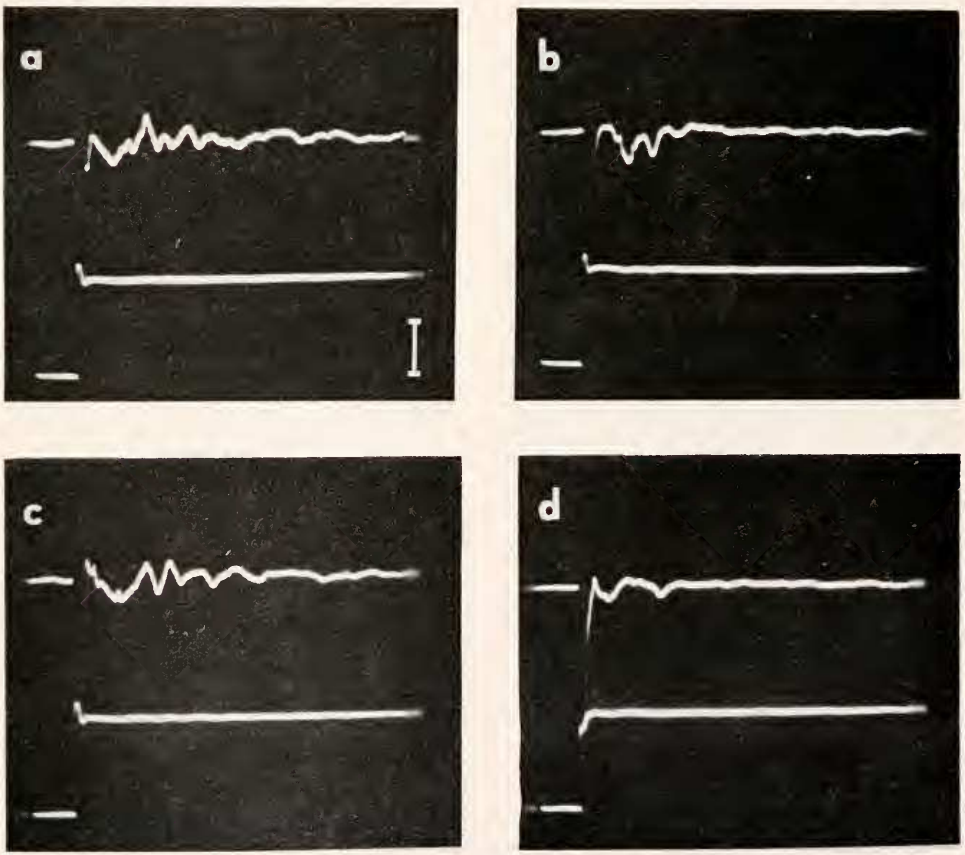

FIGURE 3. The effect of electroosmotic injection of acetylcholine on the electrical activity of the crab neuropile. Sections (A-D) represent the responses of two crabs to injection in four different areas. The top traces are the extracellularly-recorded neuropile potentials. The vertical bar represents 5 millivolts. The bottom trace shows the current record in response to a 100 volt driving pulse of 100 milliseconds duration. The pulse stopped at the end of the sweep.

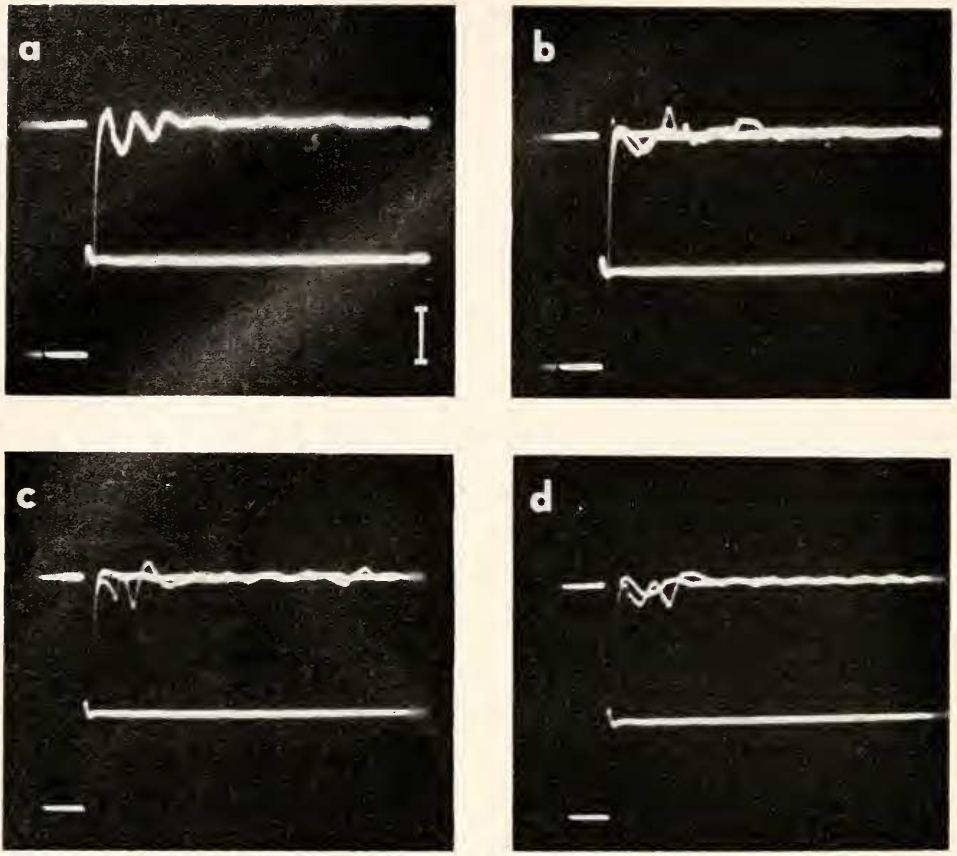

FIGURE 4. The effect of continued and repetitive injections of ACh by the electroosmotic method. In (A) the transient nature of the response, in spite of continuous injection, is shown. (B) shows the effects of three separate pulses with a repetition interval of 0.75 seconds and (C) displays two pulses. In (D) the interval was increased to 1.5 seconds.

directly ruled out, the results of this section made it unnecessary to postulate such actions.

DISCUSSION

Acetylcholine, adminstered in low concentrations by arterial perfusion to the thoracic ganglion, caused leg movements in crabs. Direct injections into the neuropile show that the effect of ACh can be attributed to its action on neural tissue. Furthermore the actions of eserine in potentiating this action of ACh, of atropine in blocking it, and the presence of desensitization and gradedness, all strongly support the contention that the ACh is acting on synaptic membrane. It might be argued that ACh is, in some manner, inducing a release of a transmitter rather than acting on synaptic membrane directly. Until there is evidence suggesting that the situation is more complex, it seems advisable to retain the simplest possible explanation.

A discussion of the experiments of earlier investigators is hampered by the diversity of preparations used and of the experimental conditions employed. Only Bonnet (1938) has reported a positive effect of ACh in low concentrations. He found that there was a short-lived enhancement of spontaneous electrical activity of the isolated ventral nerve cord of crayfish, following application of ACh in concentrations ranging from 10^{-9} to 10^{-7} g/ml. This action was followed by a depression of nervous activity. Prosser (1940) was unable to confirm Bonnet's work, finding that concentrations of ACh as large as 10^{-3} g/ml were necessary to obtain an effect.

A succession of investigations dealing with shrimp, lobsters, and crayfish also produced negative results. ACh, applied topically to the ventral nerve cord, either caused no change in spontaneous activity or, if changes were observed, the concentrations necessary to produce an effect were considered to be too large to be physiological (Ellis, Thienes and Wiersma, 1942; Turner, Hagins and Moore, 1950; Wiersma, 1952; Hichar, 1960; Welsh, 1961). I too, was unable to produce an effect in the present experiments by applying ACh topically on the thoracic ganglion. Similar difficulties have been encountered during the study of insect ganglia (Treherne, 1966).

Other investigations (Turner *et al.*, 1950; Wiersma, 1952) have employed preparations which were later shown to transmit electrically rather than by release of chemicals. The cause for a failure to find an effect of ACh in these cases is thus apparent.

The failure of ACh to act in low concentrations can be ascribed to the action of cholinesterase. In both insects and crustaceans, there is an active cholinesterase and a sizable connective tissue sheath surrounding the central nervous system (Treherne, 1966). Direct application by injection into the neuropile of crabs in the present experiments and of insects (Kerkut, Pitman and Walker, 1969; Pitman and Kerkut, 1970) has been successful in circumventing these barriers.

As I previously mentioned, in addition to the desensitization (Figure 4), the crab thoracic ganglion appears to lose sensitivity to ACh over a period of time; This phenomenon may also be responsible, at least in part, for some of the earlier failures to find a convincing effect of ACh. The mechanism of loss of sensitivity is unknown. One explanation is that perfusion may remove a substance necessary for maintenance of normal excitability. An alternative explanation is that these ganglia are sensitive to low oxygen levels and to the lack of nutrients in the perfusion fluid. Prosser and Buehl (1939)have demonstrated the importance of oxidative metabolism for electrical activity in ganglia taken from crayfish. Future experiments such as the present study must include better control of oxygenation. It is unlikely that this loss of sensitivity is a desensitization since it appears to happen without previous application of ACh.

The presence of ACh in crustacean central nervous systems has been reported by many investigators (Welsh, 1938; Jullien and Vincent, 1938; Welsh, 1939; Smith, 1939; Schallek, 1945; Tobias, Kollros and Savit, 1946; Easton, 1950; Walop, 1950; Hichar, 1960; Florey and Biederman, 1960). Furthermore both the synthesizing and degrading enzymes have been shown to be present. Choline acetylase has been found in ganglia of crayfish by Easton (1950) and of crabs by Walop (1950). The presence of cholinesterase was first shown by Marnay

and Nachmansohn (1937) for lobsters; Walop and Boot (1950) demonstrated the presence of the enzymes in *Carcinus* thoracic ganglia. E. Maynard (1964, 1971) has studied the localization of cholinesterase in lobster ventral ganglia by histochemical techniques.

A great many pharmacological studies have been performed on crustaceans using drugs known to affect cholinergic systems in other phyla. Curare injections are known to cause paralysis, depression of reflex activity, and lethargy in crustaceans (Katz, 1936; Ellis *et al.*, 1942; Wright, 1949). Atropine and eserine have also been studied. Although Prosser (1940) found no activity of atropine on the crayfish ventral nerve cord preparation, Turner *et al.*, (1950) found that it caused increased spontaneous activity and Ellis *et al.*, (1942) reported that it blocked nerve conduction. The concentrations of atropine employed were rather large. On the other hand, eserine in low concentrations (10^{-5} g/ml) has been reported to cause increased spontaneous activity (Prosser, 1940; Turner *et al.*, 1950; Hichar, 1960) and a hypersensitivity of reflex action (Wright, 1949; Florey, 1967). Easton (1957) perfused the ventral nerve cord of crayfish *via* the heart and although he was unable to demonstrate an effect of ACh alone, he found that eserine permitted actions of ACh to be seen. He observed an attenuation of reflex transmission from the legs to the abdominal muscles concerned with the tail flip. He further observed the production of slow rhythmical abdominal flexions in response to the presence of ACh in the eserine-treated preparation. However, eserine, like atropine, can cause direct effects on nerve (Ellis *et al.*, 1942) and Knowlton (1942) has found actions of high concentrations of eserine on the neuromuscular junction of a crab leg even though this is not a cholinergic system (Katz, 1936). Therefore, although the pharmacological studies involving direct application of drugs are suggestive, it is only when they are studied in conjunction with positive effects of ACh application, as in the present experiments, that they are persuasive.

A final consideration is the demonstration of a release of ACh during nervous activity. Many investigators (Welsh, 1939; Schallek, 1945; Walop, 1951) have shown that ACh is released from nervous tissue taken from crustaceans. Since there is a concomitant spontaneous electrical activity it can be said that ACh is released during nervous activity. It remains to be seen, however, if this release is correlated with the electrical activity or is merely coincidental. Walop (1950) for instance was unable to demonstrate that the release of ACh from the thoracic ganglion of *Carcinus* was augumented by electrical stimulation of the ganglion. Florey (1967) however has recently reported that perfusates of crab central nervous systems contained ACh-like activity during sensory-induced mechanical activity. His report removes an important obstacle in satisfying the criteria for ACh as a transmitter in crabs.

In summary, since ACh is present, along with enzymes for its synthesis and inactivation, and since ACh has now been shown to have an effect on the central nervous system with the attendant and expected effects of eserine and atropine, and finally, since ACh is released from crustacean nerve and ganglia in response to sensory input, it would appear safe to conclude that ACh may function as a chemical transmitter in the central nervous system of crustaceans.

Florey and Biederman (1961) suggested that ACh may be the transmitter of sensory neurons in crabs because they found little or no ACh in either the excita-

tory or inhibitory axons. ACh has long been known to be present in crustacean nerve as well as in the ganglia. Davis and Nachmansohn (1964) found that lobster leg nerve *sans* its large efferent axons, and therefore presumably entirely composed of sensory axons, was capable of synthesizing ACh. Synthesis of ACh by abdominal stretch receptors of lobsters has recently been reported by Hildebrand, Barker, Herbert and Kravitz (1971). These observations and those of Wright (1949) and of Easton (1957) which showed increased reflex responsiveness following administration of eserine, all indicate that ACh may be a transmitter in sensory neurons. However, although ACh appears to function as a chemical transmitter in sensory axons, it should be pointed out that ACh need not be the transmitter in all crustacean sensory systems nor need ACh be restricted to sensory systems. Indeed, careful experiments by Prosser (1940) showed that reflex transmission elicited by movements of caudal sensory hairs in crayfish was influenced by neither ACh nor eserine. It may also be that ACh is a transmitter of one or more interneurons.

This research was supported in part by National Institutes of Health Grant #RR7119–BSSG. I wish to thank Dr. Harry Grundfest for the generous loan of the equipment used for the electrophysiological portion of this study, and Dr. George Katz and Dr. John Reuben for valuable discussions concerning certain aspects of this study. The suggestion to administer drugs *via* arterial routes came from Dr. E. Florey.

Summary

1. The administration of acetylcholine to the thoracic ganglion of *Carcinus* by means of the sternal artery resulted in leg movements. Low concentrations of acetylcholine were effective and the action of acetylcholine was potentiated by eserine and attenuated by atropine.

2. Injection of acetylcholine into the thoracic ganglion by means of electro-osmotic injection caused electrical activity in the ganglion and desensitization was found to accompany this activity.

3. The present results and those of other investigators indicate that the classical pharmacological criteria for identification of ACh as a chemical transmitter appear to be fulfilled.

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